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Isolation of *Phaffia rhodozyma* Mutants with Increased Astaxanthin Content

GIL-HWAN AN, DONALD B. SCHUMAN, AND ERIC A. JOHNSON*

Department of Food Microbiology and Toxicology, University of Wisconsin, 1925 Willow Drive, Madison, Wisconsin 53706

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Plating of the astaxanthin-producing yeast *Phaffia rhodozyma* onto yeast-malt agar containing 50 μM antimycin A gave rise to colonies of unusual morphology, characterized by a nonpigmented lower smooth surface that developed highly pigmented vertical papillae after 1 to 2 months. Isolation and purification of the pigmented papillae, followed by testing for pigment production in shake flasks, demonstrated that several antimycin isolates were increased two- to fivefold in astaxanthin content compared with the parental natural isolate (UCD-FST 67-385). One of the antimycin strains (ant-1) and a nitrosoguanidine derivative of ant-1 (ant-1-4) produced considerably more astaxanthin than the parent (ant-1 had 800 to 900 μg/g; ant-1-4 had 900 to 1,300 μg/g; and 67-385 had 300 to 450 μg/g). The mutant strains were compared physiologically with the parent. The antimycin mutants grew slower on ammonia, glutamate, or glutamine as nitrogen sources compared with the natural isolate and also had lower cell yields on several carbon sources. Although isolated on antimycin plates, they were found to be more susceptible to antimycin A, apparently owing to the spatial separation of the papillae from the agar. They were also more susceptible than the parent to the respiratory inhibitor thenoyltrifluoroacetone and were slightly more susceptible to cyanide, but did not differ from the natural isolate in susceptibility to azide. The antimycin-derived strains were also killed faster than the parent by hydrogen peroxide. The carotenoid compositions of the parent and the antimycin-derived strains were similar to those previously determined in the type strain (UCD-FST 67-210) except that two carotenoids not previously found in the type strain were present in increased quantities in the antimycin mutants and phoenicoxanthin was a minor component. The chemical properties of the unknown carotenoids suggested that the strains isolated on antimycin agar tended to oxygenate and desaturate carotene precursors to a greater extent than the parent. The physiology of the antimycin isolates and the known specificity of antimycin for cytochrome *b* in the respiratory chain suggests that alteration of cytochrome *b* or cytochrome P-450 components involved in oxygenation and desaturation of carotenes in mitochondria are affected, which results in increased astaxanthin production. These astaxanthin-overproducing mutants and more highly pigmented derivative strains could be useful in providing a natural source of astaxanthin for the pen-reared-salmon industry or for other farmed animals that contain astaxanthin as their principal carotenoid.

The astaxanthin-containing yeast *Phaffia rhodozyma* was isolated during the early 1970s from exudates of deciduous trees in mountainous regions of Japan and Alaska (18). It was originally designated "*Rhodozyma montanae*" (18), but its unusual characteristics and the lack of a Latin description allowed the change of the genus to *Phaffia* (16), in honor of Herman Jan Phaff. *P. rhodozyma* is unusual among the pigmented yeasts because of two special properties: it ferments glucose and other sugars (compared with the other carotenoid-forming yeasts, which are all strictly aerobic) (16), and it synthesizes astaxanthin as its principal carotenoid (2). Astaxanthin is an abundant carotenoid pigment in the marine environment (24). In nature, it probably originates in certain algae, fungi, and small crustacea. These organisms start a food chain that leads to pigmentation of larger animals including salmonids (11, 12, 21, 22), crustacea (24), and birds such as the flamingo (4).

The genus *Phaffia* possesses only one species, *P. rhodozyma* (16). The yeasts are of basidiomycetous origin, but their perfect stage has not been found (16). Criteria used in identifying *P. rhodozyma* as basidiomycetous include its ability to synthesize carotenoids, its possession of certain metabolic properties such as the ability to use urea, which is less common in ascomycetous yeasts, and its cell wall structure and mode of bud formation. The most conclusive evidence of its phylogenetic origin is the multilayered cell wall near the area of repeated but formation (16), which is characteristic of heterobasidiomycetous yeasts.

Recently, there has been considerable commercial interest in using *P. rhodozyma* as a pigment source for aquacultured animals. Astaxanthin is an important constituent of aquaculture feeds because it provides necessary pigmentation, and it or precursor carotenoids may also contribute to the distinctive flavor of baked salmon (D. B. Josephson, Ph.D. thesis, University of Wisconsin, Madison, 1987.). Pigments are an expensive component of salmon diets, and some pigment sources such as shrimp shells or krill may also have detrimental properties such as high ash and bulk. Farm rearing of salmon increased from <10,000 metric tons in 1978 to >150,000 metric tons in 1987, and this output is expected to increase to >200,000 metric tons by 1990 (*New York Times*, 17 August 1987). Recently, F. Hoffmann-LaRoche Ltd. accomplished the synthesis of all-*trans* astaxanthin, which is being considered for approval as a salmonid feed additive by the Food and Drug Administration and regulatory agencies in other salmon-farming countries. However, because of the increasingly strict regulations concerning the safety of chemicals as food additives and the poor absorption of synthetic carotenoids compared with biological sources, natural carotenoid products may be given preference over synthetic

* Corresponding author.

pigments as color enhancers. *P. rhodozyma* could potentially provide a biological source of astaxanthin for pigmentation and flavor in the aquaculture industry.

In animal pigmentation experiments done with *P. rhodozyma*, high levels of astaxanthin deposition were achieved in rainbow trout, lobsters, or chicken egg yolks if the cell walls of the yeast were ruptured prior to use (7, 9, 10). The yeasts also supplied nutrients required for growth of the animals. The high lipid content of *P. rhodozyma* (10), the high efficiency of transfer of astaxanthin to salmonids in certain yeast preparations (10), and its content of carotenoids that are precursors to astaxanthin could improve both the flavor and color of commercially pen-reared salmon more effectively than a synthetic feed additive. At concentrations of astaxanthin present in natural isolates of the yeasts (300 to 450 μg of astaxanthin per g of dry yeasts), however, several percent yeasts must be added to diets to provide the 50 to 200 mg of astaxanthin per kg of feed necessary for adequate pigmentation of salmon. The relatively high requirement of yeasts adds unnecessary components to the diets including bulk cell wall polysaccharides. Strains of *P. rhodozyma* with increased contents of astaxanthin are needed to provide an economical source of astaxanthin. In this study, we describe a selection method that is useful in isolating pigmented mutants of *P. rhodozyma*.

MATERIALS AND METHODS

Chemicals. Antimycin A (a mixture of antimycins A_1 and A_3; catalog no. 2006), thenoyltrifluoroacetone (TTFA), sodium cyanide, β-carotene, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG), ethyl methanesulfonate (EMS), nystatin, all sugars except for glucose and xylose, amino acids, and hydrogen peroxide were obtained from Sigma Chemical Co., St. Louis, Mo. Glucose was from Fisher Scientific Co., Fair Lawn, N.J., and xylose was a product of J. T. Baker Chemical Co., Phillipsburg, N.J. Acetone (ACS grade) was from Fisher, and petroleum ether (residue analysis grade; boiling range, 30 to 60°C) was purchased from EM Industries, Cherry Hill, N.J. Ethanol (200 proof; USP grade) was a product of USI Chemicals, Tuscola, Ill. *trans*-Astaxanthin was a gift from Igene Biotechnology, Inc., who originally obtained it from F. Hoffmann-LaRoche Ltd. All other inorganic chemicals were purchased as the highest grades available.

Yeast strains and growth. Natural isolates of *P. rhodozyma* were kindly provided by Mary Miranda, Department of Food Science, University of California, Davis (UCD-FST). They were maintained on slants of yeast-malt medium (composed of the following [grams per liter]: glucose, 10; yeast extract, 3; malt extract, 3; Bacto-Peptone, 5 [Difco Laboratories, Detroit, Mich.]) (YM medium, Difco) with 2% agar in the refrigerator, and natural isolates and mutants were also stored in 40% glycerol–60% YM broth at −70°C.

P. rhodozyma was grown at 20 to 25°C on complex YM agar or on a defined basal medium (yeast nitrogen base medium [YNB; Difco] lacking amino acids, nitrogen, and a carbon source. The basal medium was supplemented with carbon and nitrogen sources as required (see below). Agar was added at 2% when needed. *P. rhodozyma* was also grown in 30 ml of YM broth in 300-ml baffled flasks shaken at 150 rpm on an orbital shaker controlled at 20°C. Media were unbuffered, and the initial and final pHs were 6.0 and 5.0, respectively, which was not inhibitory to yeast growth (in the presence of 0.2 and 5.0 μM antimycin, the final pHs were lowered to 4.8 and 4.0, probably owing to increased acid formation by the yeasts). We tested the influence of buffering with sodium lactate or hydrogen phthalate, but this did not significantly change the yeast or astaxanthin yields. For analysis of pigment production, yeasts were grown for 5 days in baffled shake flasks, usually in YM broth. The ability of the yeasts to use carbon sources (1% glucose, 1% cellobiose, 2% ethanol, 5% succinic acid) or nitrogen sources (ammonia, glutamate, or glutamine) was tested in YNB which was supplemented with carbon sources at 1% (wt/vol) or ammonium sulfate at 0.5%. When additional nitrogen sources were used, they were included at an equimolar nitrogen level equivalent to the quantity of nitrogen in 0.5% ammonium sulfate (0.075 M). When antimycin or TTFA was included growth media, it was first dissolved in a small quantity of ethanol (<0.1% final concentration). Growth in liquid medium was measured by determining the optical density at 660 nm; 1mg of yeasts per ml corresponds to an optical density of approximately 1.35. Growth on nitrogen sources was determined in situ in sidearm flasks with a Klett meter equipped with a red filter.

Mutagenesis. Mutagenesis of *P. rhodozyma* was attempted with UV light, EMS, or NTG. UV mutagenesis was done with cells grown in YM medium to an optical density of 0.3 to 0.4 (660 nm). Cells were aseptically poured into a petri dish in a sterile inoculating hood equipped with a UV light (General Electric germicidal UV bulb G64T6, maximum energy at 220 to 280 nm) and exposed to >95% kill (15 to 30 min depending on the strain). Cells were grown for 18 h in the dark and then plated on selective agar or YM agar.

For EMS mutagenesis, cells were suspended in 0.96 ml of phosphate buffer (pH 7.0) in 1.5-ml Eppendorf tubes. EMS (0.04 ml) was added to give a 4% concentration, and the cell suspension was mixed by vortexing and allowed to stand for 10 min. Cells were then washed four times in buffer, and thiosulfate was added in preliminary experiments to inactivate EMS. However, thiosulfate treatment was found to decrease the viability of *P. rhodozyma* and was not used in later experiments; EMS was instead removed by several water washes. Surviving cells were grown overnight before plating on YM plates and in selective media composed of YM agar plus antibiotics or other inhibitory agents (see Results).

NTG mutagenesis was done by washing freshly grown yeasts twice in 5 ml of sodium citrate (0.1 M, pH 5.5) and suspending the cells to an optical density of 1.5 to 2 (660 nm). A sample of 6.7 ml of the yeast suspension was pipetted to a culture tube, and 0.28 ml of NTG (1 mg/ml in sodium citrate) was added (final concentration of NTG, 40 μg/ml), and the suspension was vortexed for 15 to 20 s. Samples of 1 ml were transferred to sterile Eppendorf tubes, which were incubated for 20 min (>95% kill). The tubes were centrifuged, the supernatant containing the mutagen was decontaminated by treatment with hydrochloric acid according to directions provided by Sigma, and the cells were washed with 1 ml of 0.1 M potassium phosphate (pH 7.0). The yeasts were resuspended in the same buffer, and samples were inoculated into YM broth for overnight growth before plating on selective media.

Selection procedures for astaxanthin production. Because astaxanthin formation is known to be decreased by high concentrations of glucose in *P. rhodozyma* (8), we tried using 2-deoxyglucose as a selective agent to isolate glucose analog-resistant strains that might be catabolite derepressed for pigment biosynthesis. *P. rhodozyma* was very susceptible to 2-deoxyglucose; plating to YM agar containing 0.001% 2-deoxyglucose gave a >99% kill. Resistant colonies were

picked and grown in YM broth containing 8% glucose and plated to YNB plus 1% raffinose plus 0.001% 2-deoxyglucose. After two to three selections, colonies were obtained that were resistant to 0.05% 2-deoxyglucose. These were tested in YM broth for astaxanthin formation.

Several inhibitors of sterol biosynthesis (13) or carotenoid biosynthesis were incorporated in YM agar, and resistant colonies were evaluated for astaxanthin production. Inhibitors tested included ketoconazole, miconazole, 2-methylimidazole, clotrimazole, nicotine, nystatin, mevinolin, pyridine, and *N*,*N*-diethylamine. Concentrations that gave ≥90% kill were determined, and then 30 to 100 YM agar plates containing the inhibitors were inoculated with sufficient cells to give 100 to 500 surviving colonies. Colonies were visually screened for color changes.

Carotenoid extraction and analysis. After 5 days of growth in 30 ml of YM medium, the yeasts were harvested by centrifugation, washed in water, and resuspended in 30 ml of water. The optical density was measured to determine growth. Approximately 13 ml of the suspension was broken with the same volume of 0.5-mm glass beads for 3 to 4 min with cooling in a Bead Beater (Bio Spec Products, Bartlesville, Okla.). After breakage, the bead-cell mixture was poured into a beaker and extracted five times with 10-ml aliquots of acetone. The acetone extracts were pooled and centrifuged at 27,000 × *g* for 45 min. The clear acetone supernatant was poured off the cell pellet; if the pellet contained residual pigment, it was manually ground with a glass homogenizer and further extracted with acetone. The combined acetone extracts were combined in a separatory funnel, and approximately 10 ml of petroleum ether was added as well as a few milliliters of a solution of saturated NaCl to help break emulsions. The petroleum ether extract was collected after removal of the acetone layer, which was reextracted. The petroleum ether extracts were combined and were usually filtered through glass wool to remove lipid globules and other particulate matter. The absorbance spectrum was recorded on a Gilford response spectrophotometer (the absorbance maximum of *trans*-astaxanthin in petroleum ether is 474 nm). The total carotenoid composition was calculated by using the 1% extinction coefficient = 2,100 by the formula:

$$\text{Total carotenoid } (\mu g/g \text{ of yeasts}) = \frac{(\text{ml of petrol})(A_{474})(100)}{(21)(\text{yeast dry weight})}$$

Growth and extraction of yeast strains were repeated at least twice in independent trials to accurately assess carotenoid production.

Individual carotenoids were analyzed by thin-layer chromatography (TLC) and electronic absorption spectra. Cells were grown for 3 to 4 days in YM broth at 20°C. To prepare carotenoid extracts for chromatography, petroleum ether partitions were dried over anhydrous $CaCl_2$ and concentrated by evaporation in a stream of nitrogen. These were chromatographed by TLC on silica gel plates (Silica Gel 60, 5 by 20 cm, 0.25 mm thickness; E. Merck AG, Darmstadt, Federal Republic of Germany) with 20% acetone–80% petroleum ether. After development, bands were scraped and eluted in acetone through a Pasteur pipette plugged with glass wool. Absorbance maxima (2, 3), R_f values (3), and cochromatography with standards (β-carotene and *trans*-astaxanthin) were used for identification of the pigments. Carotenoid concentrations were calculated by the extinction coefficients listed by Davies (3). Astaxanthin was isomerized by dissolving *trans*-astaxanthin in equal parts of acetone-methanol (0.1 mg/ml), adding 2% iodine, and exposing the solution to fluorescent light for 3 h (26). TLC was then done to determine the R_f of *cis*-astaxanthin.

RESULTS

Comparison of natural isolates. Most previous work with *P. rhodozyma* has been done with the type strain, UCD-FST 67-210. Visual examination of young colonies of several additional natural isolates (UCD-FST 62-202, 67-203, 67-210, 67-383, 67-385, 67-384, and 68-653C) indicated that 67-210, 67-384, and 67-385 were the most highly pigmented strains. Quantitative determination of total carotenoid (estimated as astaxanthin content) in these three strains after 5 days of growth in YM broth indicated that 67-385 contained ~450 μg/g of dry yeasts compared with ~295 μg/g in 67-210 and ~280 μg/g in 67-384. Extraction of 67-202, a visually low producer, indicated that it produced 202 μg of astaxanthin per g but had a high proportion of carotenes. We used 67-385 for strain development because of its higher natural astaxanthin content.

Mutagenesis of *P. rhodozyma*. We evaluated the effectiveness of UV light, EMS, and NTG in generating pigmented mutants. The natural isolate 67-202, which has approximately 50% less astaxanthin than 67-385 but may contain a higher content of carotenes, survived better than 67-385 during UV exposure. This result suggested that carotenoids react with UV (15); however, attempts at isolating highly pigmented variants were not successful after screening of several thousand colonies following UV exposure. Most of the UV-generated mutants were reduced in astaxanthin content and were very pale. EMS mutagenesis generated some strains (~0.2% frequency) that contained ~50% more astaxanthin than the parent when tested in shake flasks (e.g., 600 μg/g compared with 400 μg/g in the parent strain 67-385). A second mutagenesis of selected EMS mutants resulted in a high frequency of reversion (20 to 40%) to the color of the parental colonies and a much lower frequency (~0.5%) of colonies that were pinker. However, these mutants were often very unstable, and 5 to 10% of the population reverted after several successive transfers in YM broth. NTG was the best mutagen tested; its use generated considerable variation in pigmentation among the colonies screened. Purified strains still often reverted at high frequency, but stable clones could be obtained by repeated mutagenesis with NTG.

Selection procedure. Since screening of colonies after mutagenesis was relatively unsuccessful in finding pigmented strains, we tried to develop selection procedures for astaxanthin overproduction. Selection for resistance to 2-deoxyglucose (see Materials and Methods) was not successful in isolating highly pigmented mutants. Although some strains generated by this selective procedure were changed in pigmentation, many were unstable and none were increased in astaxanthin concentration more than twofold over the concentration found in 67-385. After several months, we tried alternative selective methods.

Similarly, *P. rhodozyma* strains resistant to sterol biosynthesis inhibitors (see Materials and Methods) did not yield high-producing strains. Several of the inhibitors tested including nicotine (1 mM), imidazole (4 mM), 2-methylimidazole (1 mM), and morpholine (10 mM) changed the color of the colonies distinctly, indicating a change in carotenoid composition. However, since the visual appearance or absorbance spectra of extracts did not resemble astaxanthin,

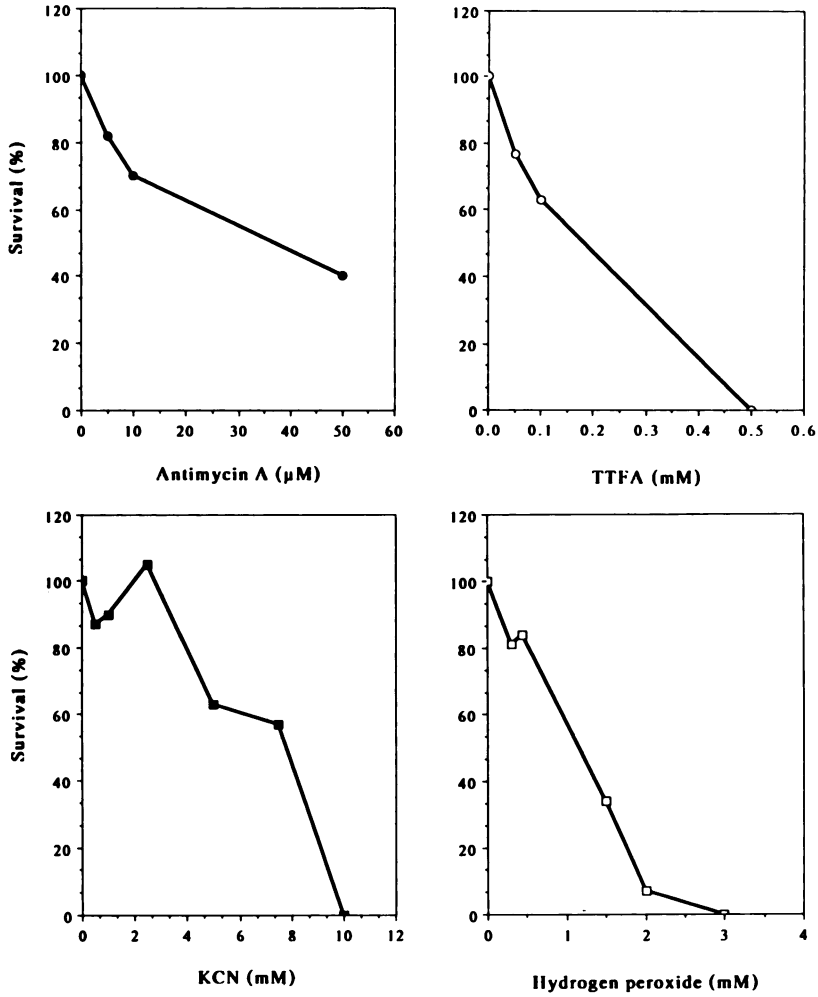

FIG. 1. Susceptibility of *P. rhodozyma* wild strain (UCD-FST 67-385) to antimycin A, TTFA, sodium cyanide, and hydrogen peroxide.

we did not analyze the carotenoids produced in the presence of the inhibitors.

It was shown previously that high levels of glucose or reduced oxygen availability significantly reduced astaxanthin biosynthesis and caused accumulation of carotene pigments (8). These results suggest that oxygenation of the carotenes through activation of molecular oxygen is rate limiting for astaxanthin synthesis in some growth environments. By analogy to other related pathways, e.g., sterol biosynthesis, the source of active oxygen and oxygenating enzymes would likely be the mitochondrial respiratory chain and associated heme proteins (e.g., cytochrome P-450) (6, 17). Therefore, we tried treating *P. rhodozyma* with several inhibitors of electron transport including TTFA, antimycin A, potassium cyanide, sodium azide, and hydrogen peroxide. *P. rhodozyma* UCD-FST 67-385 was killed by low concentrations of antimycin A, TTFA, KCN, and H_2O_2 (Fig. 1) but was relatively insensitive to azide (data not shown). We plated several thousand cells of *P. rhodozyma* on YM agar containing 50 μM antimycin. Colonies required 1 to 2 months to develop fully and took on a very peculiar morphology (Fig. 2). Pale, smooth colonies initially grew closest to the agar, but after several weeks these primary colonies gave rise to highly pigmented vertical growths. We retested various concentrations of antimycin A for generating varie-

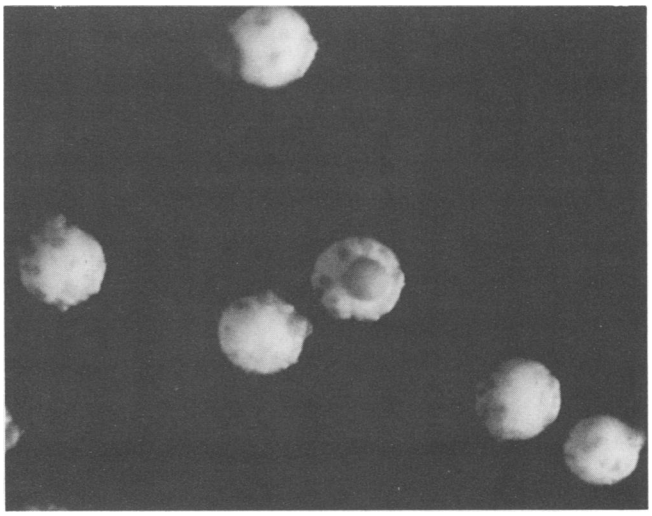

FIG. 2. Colonies of *P. rhodozyma* UCD-FST 67-385 after 1 month on YM agar containing 50 μM antimycin A.

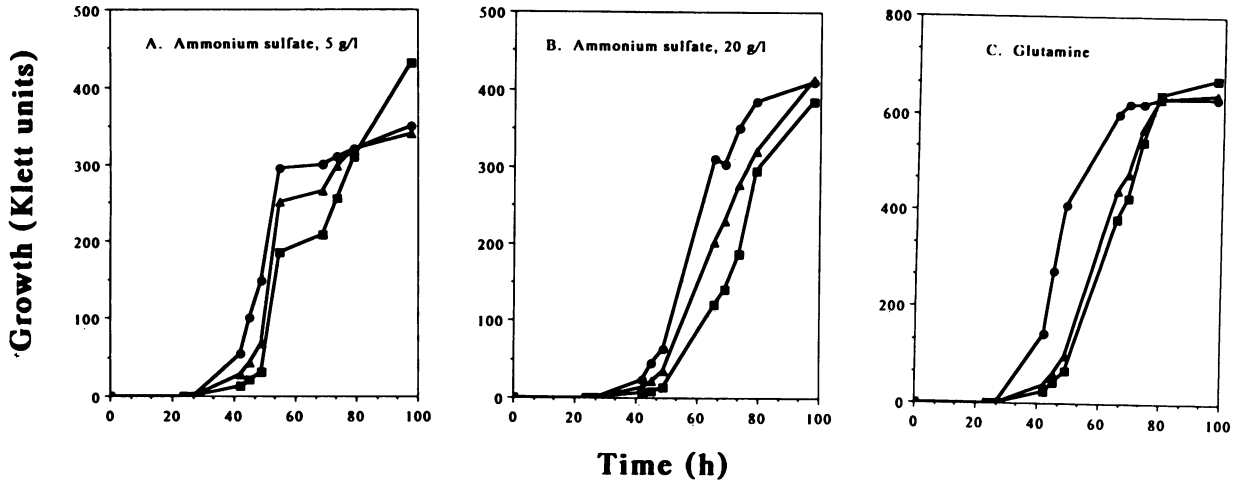

FIG. 3. Growth of *P. rhodozyma* on various nitrogen sources.

gated colonies (1 to 100 μM antimycin in YM agar), and the optimum concentration for generation of the colonies with the most highly pigmented protrusions was 50 μM. The highly pigmented papillae also seemed to arise most frequently when the developing colonies were exposed to aerobic conditions after being incubated for 1.5 months tightly sealed in the petri dish plastic sleeve. One of the papillae was dissected from a basal colony (ant-1) and contained approximately 900 μg of total carotenoid per g of yeasts (estimated as astaxanthin) compared with the parental strain, which had 300 to 400 μg/g. This mutant was further treated with NTG, and a derivative strain (ant-1-4) was isolated that produced 1,200 to 1,500 μg of total carotenoids per g of yeasts. However, this mutant, ant-1-4, was less stable than ant-1, and repeated transfer on YM slants reduced its carotenoid titer to 900 to 1,200 μg/g of yeasts compared with ant-1, which did not decrease in carotenoid titer on subcultures.

We also replated ant-1 to YM agar containing 50 μM antimycin and selected colonies that were smaller (1 to 2 mm) compared with the majority of the colonies, which were larger (2 to 3 mm). These second-generation antimycin mutants did not form papillae. However, some of the colonies were increased in pigmentation. One of the mutants produced 1,200 μg/g (dant-1), and another produced 1,450 μg/g (dant-2). Therefore, it appears that antimycin A is an excellent selective agent for isolating pigmented variants of *P. rhodozyma* by direct selection.

After finding that mutants susceptible to antimycin were often increased in astaxanthin, we tested some of the EMS-generated mutants that were previously isolated without antimycin but were kept because of their increases in pigmentation. Two of the more highly pigmented mutants tested (27 and 18) were more susceptible to antimycin; therefore, it seems that the changes induced by antimycin involve alteration of an important control in astaxanthin biosynthesis and that susceptible strains can be isolated by several independent methods not requiring antimycin.

Physiological characterization of antimycin mutants. The parental strain (67-385) and the mutants ant-1 and ant-1-4 gave approximately equal final weights of cells after 5 days of growth in shake flasks with ammonium or glutamine as the nitrogen source (Fig. 3). However, the mutant strains grew slower on each nitrogen source (Fig. 3). Growth of the mutants was also slower when glutamate was used as the source of nitrogen, although the difference from the control was not as large as observed for glutamine (data not shown). The growth rate of the mutants did not improve significantly when the ammonia concentration was raised fourfold to 20 g/liter (Fig. 3). It appeared that nitrogen utilization was impaired in the antimycin mutants.

The two studied mutants had reduced cell growth compared with the parent on four carbon sources tested (Table 1). The mutants were more highly pigmented on each of the energy sources. The antimycin-derived mutants fermented glucose and also had significant ethanol dehydrogenase activity (data not shown). However, ant-1-4 did not grow as well as the parent on ethanol and gave reduced cell yields on succinate, which is another respiratory substrate. The reduced yields on the energy sources, together with the specificity of antimycin for the respiratory chain, suggested that mitochondrial function in the pigmented mutants was altered.

We examined susceptibilities of the mutants to various respiratory inhibitors or iron-reactive compounds including antimycin A, TTFA, sodium azide, hydrogen peroxide, and

TABLE 1. Growth and pigmentation of *P. rhodozyma* and antimycin mutants on carbon sources

Yeast strain	Growth (mg/ml) on the following carbon source (2%):				Total carotenoid (μg/g of yeasts) after growth on:			
	Glucose	Cellobiose	Ethanol	Succinate	Glucose	Cellobiose	Ethanol	Succinate
67-385	2.57	3.92	2.25	0.10	290	270	540	ND[a]
ant-1	2.01	4.11	2.10	0.06	670	300	380	ND
ant-1-4	1.82	1.78	0[b]	0.04	1,050	1,260	ND	ND

[a] ND, Not determined.
[b] Grows weakly on lower concentration of ethanol (<1%).

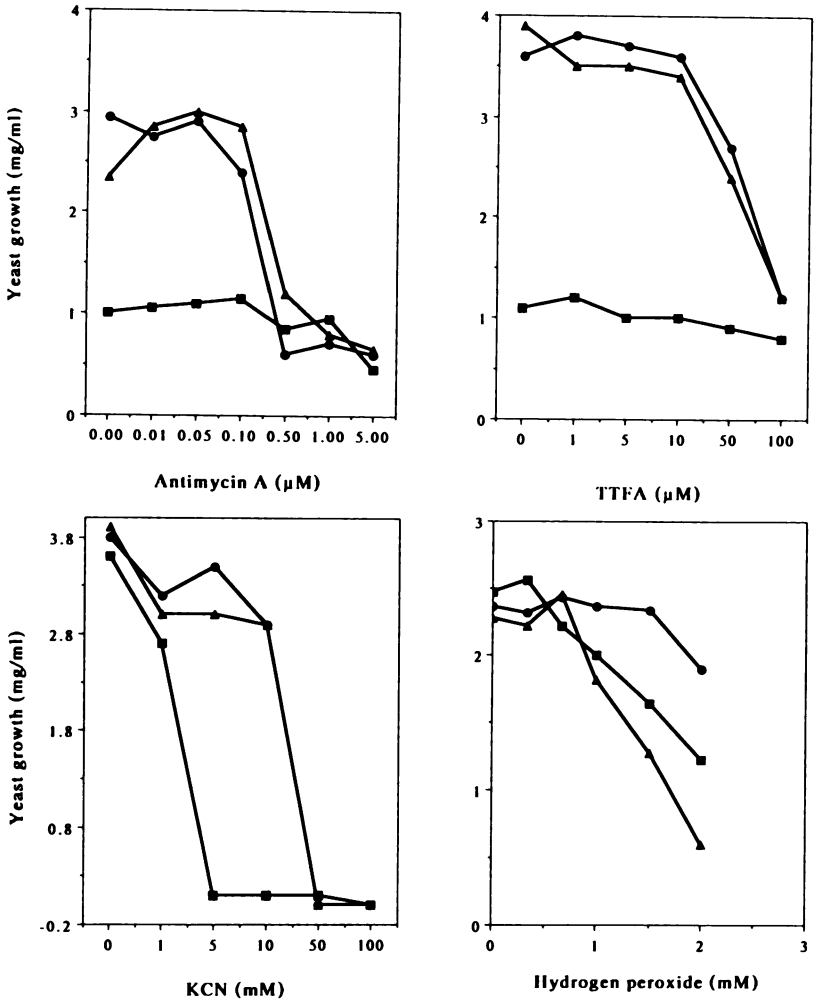

FIG. 4. Susceptibility of *P. rhodozyma* and antimycin mutants to respiratory inhibitors. Symbols: ●, UCD-FST 67-385 (natural isolate); ▲, ant-1; ■, ant-1-4.

KCN. Surprisingly, we found that the antimycin-induced mutants were more susceptible to antimycin A on YM plates (Fig. 4) and in liquid media, although they were isolated in selections for resistance. On YM agar, the parental strain had approximately 50% survival at 100 μM antimycin compared with the mutants, which did not survive above 60 μM. The concentrations of antimycin that killed 50% of the populations of ant-1 and ant-1-4 on YM agar were approximately 18 and 3 μM, respectively. The mutants were also more susceptible in liquid media and were killed at antimycin concentrations near 0.5 μM. These results clearly show that the pigmented papillae that arose from the pale colonies were actually more susceptible to the drug even though they were isolated on plates containing antimycin. Apparently, the spatial separation of the papillae from the agar allowed the generation of more susceptible strains.

The mutants were susceptible to cytochrome inhibitors other than antimycin including TTFA, hydrogen peroxide, and cyanide (Fig. 4). However, they did not differ significantly in susceptibility to azide (data not shown). These data further supported the hypothesis that the antimycin-isolated strains possessed an altered respiratory chain and seem to indicate that the lesions(s) occurs in the early regions of the chain, probably near cytochrome *b*, the site of antimycin activity.

Carotenoid compositions of mutants. We examined the carotenoids in the mutants and in the wild-type parent and found that the pigment contents differed (Table 2). Based on R_f values by TLC and electronic absorption spectra (2, 4), the antimycin-induced strains appeared to have increased concentrations of two carotenoids (unknowns 1 and 2) that were present in very low quantities in the parental natural isolate and were not previously reported in strain UCD-FST 67-210 by Andrewes et al. (2). On the basis of polarity during TLC and the electronic absorption spectra (Table 3), the unknown carotenoids in the mutants possibly had the structures 3,3′-dihydroxy-β-Ψ-caroten-4, 4′-dione (DCD) and 4-hydroxy-3′,4′-didehydro-β-Ψ-carotene (HDC). Unknown 1 (DCD) was more polar than *cis*-astaxanthin (Table 3) and had an absorption spectrum with bands at 461 nm (shoulder), 490 nm, and 523 nm. Unknown 2 (HDC) was slightly more polar than 3-hydroxyechinenone and showed an absorption spectrum with a shoulder at 464 nm, and peaks at 489.5 and 521 nm. Spectra and chromatographic properties similar to those of DCD have been described by Reichenbach and Kleinig (19) and Aasen and Liaaen-Jensen (1) for *trans*-3,4-dehydrolycopene. HDC was also found in flexibacteria (1) and was given the common name anhydro-deoxy-flexixanthin. Further experiments including isolation and spec-

TABLE 2. Comparison of primary carotenoids in *P. rhodozyma* grown in the presence of antimycin

Carotenoid	% of total carotenoids at the following antimycin concn (μM):								
	67-385			ant-1			ant-1-4		
	0	0.1	10	0	0.1	10	0	0.1	10
Unknown 1	ND[a]	ND	ND	2	1	ND	4	ND	ND
cis- and *trans*-Astaxanthin[b]	60	71	70	78	63	23	55	66	47
Phoenicoxanthin	5	ND	3	ND	ND	3	2	ND	5
3-Hydroxy-3′,4′-didehydro-β-Ψ-carotene-4-one	7	16	8	10	19	9	25	22	15
Unknown 2	2	3	3	2	6	4	6	6	ND
3-Hydroxy-echinenone and echinenone	10	3	8	2	4	25	4	3	16
β-Zeacarotene	ND	2	ND	ND	1	ND	1	1	ND
β-Carotene	10	3	6	1	2	30	2	2	15
Other carotenes	8	3	3	5	5	7	3	2	5

[a] ND, None detected.
[b] Approximately 10% *cis* form.

troscopy will be needed to unequivocally identify the unknowns in *P. rhodozyma* 67-385.

In comparison with the natural isolate, the antimycin-susceptible mutants also had slightly increased concentrations of astaxanthin and 3-hydroxy-3′,4′-didehydro-β-Ψ-caroten-4-one and lesser quantities of phoenicoxanthin, echinenone, 3-hydroxyechinenone, and β-carotene (Table 2). ant-1-4 formed mostly *trans*-astaxanthin but also produced low quantities of *cis*-astaxanthin that were detectable by iodine isomerization (26).

We investigated the changes in carotenoid composition when 0.1 or 10 μM antimycin was added to 27-h-old cells in the log phase in YM broth. The carotenoid composition changed significantly in *P. rhodozyma* (Table 4). For both the parent and the mutants, the proportions of several pigments were increased including astaxanthin, 3-hydroxy-3′,4′-didehydro-β-Ψ-caroten-4-one, and β-carotene. In the mutants, but not in the parent, increased proportions of the two unknowns were detected and echinenone and 3-hydroxyechinenone were also detected. In the parent, phoenicoxanthin, which is the second most predominant carotenoid in UCD-FST 67-210 (1), was no longer detected.

Our results indicated an apparent discrepancy from the carotenoid analysis originally done by Andrewes et al. (2), who showed that phoenicoxanthin was the second most abundant carotenoid in UCD-FST 67-210. Our results indicated that the quantity of phoenicoxanthin was comparatively low in UCD-FST 67-385 and its antimycin mutants. To determine whether the discrepancy was due to a difference in strains, culture conditions, or analytical methods, we compared the pigments previously characterized in 67-210 by Andrewes et al. (2) with the composition of our mutants and also determined the carotenoid composition of ant-1 at various times following inoculation (Table 4). The results showed that phoenicoxanthin was more prevalent in 67-210 than in ant-1; however, we also detected low quantities of unknowns 1 and 2 in 67-210. Therefore, culture conditions could have contributed to the absence of the pigments in the original analysis by Andrewes et al. (2), who grew the yeasts on agar plates and scraped the cells off with a microscope slide; in contrast, we grew *P. rhodozyma* in broth in shake flasks. The time course of pigment formation over an 18-day period showed that unknown 1 increased four- to five-fold, *cis*-astaxanthin increased about threefold, and 3-hydroxyechinenone decreased two- to threefold, while the proportions of the other pigments remained relatively constant throughout the 18-day incubation.

TABLE 3. Properties of the carotenoids in *P. rhodozyma* grown in the presence of antimycin

Carotenoid	Absorbance maximum (nm)[a]			R_f[b]
Unknown 1	(461)	490	523	0.19
cis-Astaxanthin	366	470		0.22
trans-Astaxanthin		477		0.29
Phoenicoxanthin		474		0.45
3-Hydroxy-3′,4′-didehydro-β-Ψ-caroten-4-one	(463)	493	523	0.62
Unknown 2	(464)	489	521	0.68
3-Hydroxyechinenone		464		0.71
Echinenone		460		0.73
γ-Carotene	438	464	496	0.89
β-Zeacarotene	402	427	448	0.93
β-Carotene	429	452	481	0.95

[a] Spectra recorded in acetone.
[b] TLC solvent system was 20% acetone–80% petroleum ether on Silica Gel 60, 0.25 mm thickness.

TABLE 4. Carotenoid compositions of *P. rhodozyma* 67-210 and ant-1[a]

Carotenoid	μg of carotenoid/g of yeasts after the following days of incubation:			
	67-210 (3.5)	ant-1[a]		
		3.5	8	18
Unknown 1	6	14	32	116
cis-Astaxanthin	4	28	64	140
trans-Astaxanthin	167	567	658	932
Phoenicoxanthin	20	14	11	17
3-Hydroxy-3′,4′-didehydro-β-Ψ-caroten-4-on	21	126	128	239
Unknown 2	10	65	64	74
3-Hydroxyechinenone	24	37	37	25
Echinenone	1	5	5	8
γ-Carotene	0	5	5	17
β-Zeacarotene	0	5	5	8
β-Carotene	25	14	16	8
Others	17	51	43	66

[a] ant-1 is derived from 67-385 as described in the text.

DISCUSSION

The present report describes a selection procedure that is effective for isolating strains of *P. rhodozyma* with significantly higher concentrations of astaxanthin. We found that pale, smooth colonies initially form on antimycin A-containing plates after 3 to 5 days of incubation, but after 1 to 2 months they give rise to bulbous, highly pigmented vertical growths. These peculiar growths were dissected from the colonies and were characterized physiologically. They grew well in minimal medium (YNB) and were not auxotrophic for any growth factors as far as we could discern. However, they did show slower growth on various nitrogen sources and had decreased yields on carbon sources. The basis for slower growth on nitrogen sources is not currently understood. The reduced yields on carbon sources suggest that the antimycin mutants are not obtaining equivalent quantities of energy from the carbon as the parents. This datum suggests that the highly pigmented mutants have an altered respiratory chain. Choosing small colonies on second-round selection on antimycin plates also yielded mutants with higher pigmentation. The small size and slower growth observed in YM broth supported the hypothesis that increased susceptibility to antimycin led to impairment of energy acquisition.

Antimycin specifically inhibits the respiratory chain between cytochromes *b* and c_1 (6, 20). Spectral analyses of the inhibited chain under steady-state conditions have shown that cytochromes b_{566} and b_{562} exhibit unexpected redox behavior: oxidation of cytochrome *c* promotes the reduction of the cytochromes *b*, particularly b_{566}, and reduction is stimulated by antimycin A (25). Addition of antimycin to respiring mitochondria causes an increased absorption that is due mostly to increased reduction of cytochrome b_{566} (25). Accumulation of reduced cytochromes *b* could promote increased reduction of cytochrome P-450 through the activity of NADPH-cytochrome P-450 reductase with a consequent increase in hydroxylation and desaturation of carotenoids (23). The transfer of reducing equivalents could be accelerated in our mutants owing to slower nitrogen utilization in the presence of rapid carbon catabolism. Under these conditions, it would be expected that the excess NADPH normally used for amino acid, nucleotide, and other biosyntheses could be disposed of by the electron transport chain. However, if there is an alteration at cytochrome *b*, then there could be a tendency to dispose of the hydrogen through NADPH-cytochrome *b* reductase coupled to an outlet such as cytochrome P-450. Cytochrome P-450 has been demonstrated to be involved in several steps in sterol biosynthesis in *Saccharomyces cerevisiae* and other yeasts (5, 6, 23). The P-450 system is involved in demethylation, desaturation, and oxygenation of sterol intermediates (6, 23). Certain cytochromes P-450 have been postulated to have coevolved with sterol metabolism (17). Our search of the available literature did not give evidence that cytochrome P-450 is involved in carotenoid oxygenation of desaturation, although its involvement in similar biosynthetic reactions in sterol biosynthesis would imply that it could also be involved in carotenoid transformations. In general, the antimycin-susceptible mutants appeared to hydroxylate and desaturate carotenoid intermediates more readily as shown by analysis of the pigments, but appeared less prone to carry out ring closure. These reactions are known to be carried out by cytochrome P-450 in sterol and steroid biosynthesis in yeasts and other organisms (5, 6, 14, 23).

The composition of certain of the carotenoids in the antimycin mutants isolated in this study is markedly similar to that of the pigments previously analyzed in *P. rhodozyma* cultured under microaerophilic conditions (8). The cells in both cases tend to accumulate higher quantities of carotenes and also to synthesize β-zeacarotene, which is not ordinarily produced by *P. rhodozyma* under optimal growth conditions. Since cytochrome P-450 in yeasts is most highly expressed under microaerophilic conditions (13), it seems possible that our mutants are altered in their activity or regulation of this cytochrome and its reducing system. In conclusion, the mechanism by which antimycin selection increases astaxanthin formation in *P. rhodozyma* is not known, but it appears that reactions associated with the respiratory chain and possibly cytochrome P-450 are important in controlling the rate of astaxanthin biosynthesis.

Antimycin seems to induce synthesis of carotenoids not present in significant quantities in the wild-type yeasts. The two unknowns formed in the presence of antimycin may correspond to DCD and HCD from the analysis of their chromatographic properties and electronic absorption spectra. The determination of their unequivocal structures will require further characterization. The increased quantities of astaxanthin produced by the antimycin mutants suggests that these unknowns still serve as precursors to astaxanthin. If they are transformed to astaxanthin, this would suggest that the order of enzymatic transformations at the end regions of the carotenoids, e.g., cyclization, desaturation, and oxygenation, does not occur in a consistent order. Possibly an enzyme complex is responsible for binding the lipid and catalyzing several reactions. Although we have not extensively looked for mutants blocked at individual steps, we have been able to isolate mutants blocked at β-carotene but not at the xanthophyll intermediate biosynthetic steps. Also, physiological experiments have shown that carotenes but not xanthophylls accumulate under conditions of stress such as low pH or microaerophilic conditions (8). These findings support the possibility that an enzyme complex is involved in xanthophyll generation. This hypothesis can only be tested by studies of the biosynthetic enzymes and genetics of astaxanthin formation.

The antimycin-susceptible mutants isolated in this study were useful as parental strains to isolate strains further increased in astaxanthin formation. Two successive mutagenic steps of ant-1-4 yielded strains that consistently produce >2,000 μg of astaxanthin per g of yeasts; and an independent laboratory (B. Flenoe, Danisco Biotechnology, personal communication) has reported yields in excess of 2,500 μg/g with the same strains. These mutants have been used for salmon feeding trials and have effectively imparted color into the fish (W. T. Hall, Igene Biotechnology, Inc., personal communication). The astaxanthin-overproducing mutants further derived from the antimycin strains described in this study are presently being characterized in our laboratory.

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